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# Intramolecular Thioether Crosslinking of Therapeutic Proteins to Increase Proteolytic Stability

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Protein-based pharmaceuticals typically display high selectivity and low toxicity, but are also characterized by low oral availability, mainly because of enzymatic degradation in the gastrointestinal tract and poor permeability across the intestinal wall. One way to increase the proteolytic stability of peptides and proteins is by intramolecular crosslinking, such as the introduction of disulfide bridges. However, disulfide bridges are at risk of thiol-disulfide exchange or reduction during production, purification, and/or therapeutic use, whereas thioether bridges are expected to be stable under the same conditions. In this study, thioether crosslinking was investigated for a 46 aa albumin-binding domain (ABD) derived from streptococcal protein G. ABD binds with high affinity to human serum albumin (HSA) and has been proposed as a fusion partner to increase the in vivo half-lives of therapeutic proteins. In the study, five ABD variants with single or double intramolecular thioether bridges were designed and synthesized. The binding affinity, secondary structure, and thermal stability of each protein was investigated by SPR-based biosensor analysis and CD spectroscopy. The proteolytic stability in the presence of the major intestinal proteases pepsin (found in the stomach) and trypsin in combination with chymotrypsin (found in pancreatin secreted to the duodenum by the pancreas) was also investigated. The most promising crosslinked variant, ABD_CL1, showed high thermal stability, retained high affinity in binding to HSA, and showed dramatically increased stability in the presence of pepsin and trypsin/chymotrypsin, compared to the ABD reference protein. This suggests that the intramolecular thioether crosslinking strategy can be used to increase the stability towards gastrointestinal enzymes.

## Introduction

Protein-based therapeutics have demonstrated increased clinical importance during recent years because of their excellent properties such as high selectivity and potency. The high specificity tends to result in lower risk of severe side effects and thus faster clinical development and approval times compared to low-molecular-weight drugs.[1–3] However, protein and peptide therapeutics have a few shortcomings, such as high production cost and risk of immune response.[4] Another drawback is the need for injection or infusion for administration, and considerable effort has been directed to the development of orally available therapeutic peptides and proteins.

The first important degradation step to overcome is associated with the low pH and the digestive enzyme pepsin in the stomach. Later, in the duodenum, the drug is challenged by additional proteases, including trypsin and chymotrypsin found in pancreatin (the fluid secreted by the pancreas).[5–7] Avoidance of degradation by these three enzymes and tolerance to low pH are important for an orally available protein-based drug.

A second obstacle is poor absorption in the intestine, as proteins generally have hydrodynamic radii that are too large to pass through the tight junctions. However, with the use of different formulations and additives, the uptake of small- to medium-sized proteins such as insulin (5 kDa) has been reported.[5,6,8,9] Other possible administration routes are pulmonary and nasal delivery.[10,11]

Small, robust proteins can potentially be engineered to withstand the rough conditions of the gastrointestinal tract and to be small enough to be absorbed into the bloodstream. Naturally occurring stable proteins such as cyclotides and cysteine knot mini-proteins have been carefully studied and engineered, and show very promising results for oral administration.[12–17] A common characteristic of these is intramolecular crosslinking, a feature also found in the peptide hormones oxytocin and somatostatin.[18,19] Cyclic structures tend to be more stable toward proteolytic degradation compared to their linear counterparts because of the absence of free N and C termini (susceptible to exoproteases) and the reduced access of endoproteases to proteolytic sites in the conformationally rigid structures.

Stabilization by the introduction of thioether bridges has also shown promising results for peptides lacking a cyclic backbone or intramolecular crosslinks.[20,21] As the thioether bond is resistant to reduction and thiol–disulfide exchange, this type of bridge can be expected to yield peptides and proteins with higher in vivo stability compared to the disulfide-linked variants.[22] Thioether crosslinks are generally more difficult to introduce into proteins than into short peptides, but incorporation can be achieved by enzymatic modification or chemical strategies such as alkylation of cysteine residues by crosslinking agents.[23–25] However, for small (< 80 aa) proteins, produc-

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tion by solid phase peptide synthesis is feasible. Thioether modification by chemical synthesis and orthogonal protection is straightforward and can be achieved by, for example, the introduction of appropriately positioned thiol and haloacetyl groups.[26] Synthetic strategies offer new possibilities (as yet, not extensively explored) to engineer small, therapeutic proteins with increased stability.

In this work we used a 46 aa albumin-binding domain (ABD),[27,28] derived from the third ABD from streptococcal protein G ("G148-GA3"), as a model to study the effect of intramolecular thioether bridges on the stabilization of small, therapeutic proteins. ABD binds with very high affinity to human serum albumin (HSA) and has shown therapeutic relevance by extending the half-lives of drugs.[29–31] The very long in vivo circulation half-life is probably a result of high-affinity binding to albumin, thereby resulting in the formation of a tight complex in vivo.[30,32] The long circulation half-life, comparable to albumin itself, is a strong indication of the very high serum stability of ABD. However, as demonstrated here, the protein is sensitive to degradation by gastrointestinal proteases. Five crosslinked variants of ABD were synthesized and evaluated for albumin binding, secondary structure, and thermal stability. Stability in the presence of the digestive enzymes pepsin and a trypsin/chymotrypsin combination was investigated; the most promising variant was studied in detail and compared to an ABD reference protein.

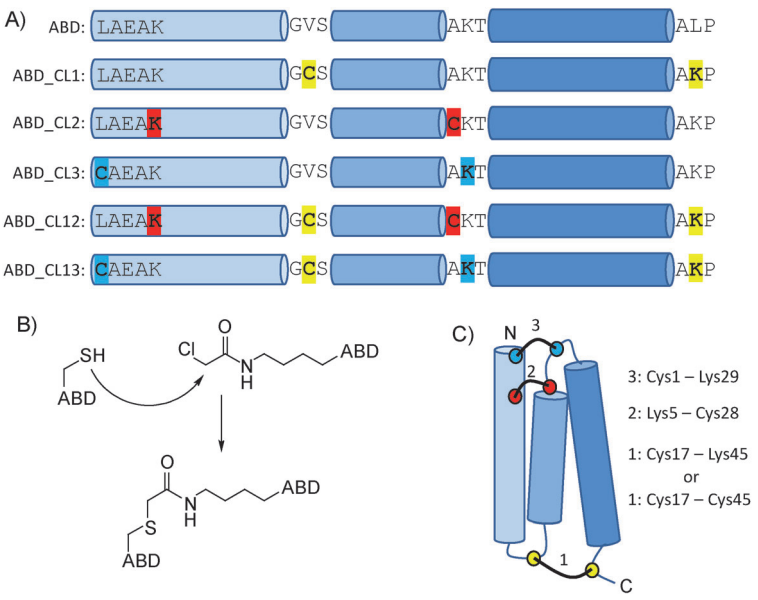


**Figure 1.** Design of thioether-crosslinked proteins. A) The three helical regions, with the crosslinking amino acids highlighted. B) Thioalkylation reaction between the side chain of cysteine and the chloroacetylated side chain of lysine. C) The three-helix protein, based on the NMR structure of a homologous domain (ref. [33]), with the three crosslinking sites indicated by colored circles and solid lines.

## Results

### Peptide synthesis and intramolecular crosslinking

The ABD proteins were synthesized and functionalized with chloroacetyl groups at selected lysine residues (Figure 1 and Figure S1 in the Supporting Information). The ABD proteins were designed such that each functionalized lysine residue had a partner cysteine in close proximity when the protein is folded in the expected three-helix bundle, based on the three-dimensional structure solved for the G148-GA3 domain (PDB ID: 1GJT).[33] The sites for crosslinking were selected based on the NMR structure of this domain, and chosen to form links from the N-terminal part of the protein to the loop region between helices two and three, and/or from the C-terminal part of the protein to the loop region between helices one and two. Based on the 3D structure, the chosen residues should be well positioned for the tethering by simply adding a chloroacetyl group to the side chain of a lysine residue, thus allowing reaction with the thiol of an introduced cysteine residue. The calculated distances, based on the homologous domain, were in relatively good agreement with the calculated linker lengths ($C^{\alpha}$ of a lysine residue to $C^{\alpha}$ of cysteine linked by an acetyl group: ~12 Å). In the homologous ABD structure the $C^{\alpha}$–$C^{\alpha}$ distances between potentially crosslinked residues are 11.1 Å between residues 17 and 45 (linker 1), 7.8 Å between residues 5 and 28 (linker 2), and 12.6 Å between residues 1 and 29 (linker 3).

The crosslinking reaction was performed by dissolving the protein in buffer, under conditions such that the thiol group of the cysteine residue can make a nucleophilic attack on the $\alpha$-carbon of the chloroacetyl group, thus forming a thioether bond. For three ABD proteins with one functionalized lysine (ABD_CL1, ABD_CL2, and ABD_CL3; Figure 1 A) the crosslinking reaction proved to be very efficient, without competing side reactions. When using crude, unpurified ABD in the reaction, the correct crosslinked product was obtained in relatively high synthetic yield (10–17%, based on the HPLC elution profile at 220 nm; Figure 2). The crosslinking of ABD_CL12 and ABD_CL13 (two functionalized lysine residues) proved to be more complex. The expected molecular weight of the double-crosslinked protein was found in two separate HPLC fractions, which were assayed separately and in later analyses showed similar properties. No disulfide-linked dimers or intramolecular disulfides were identified after the crosslinking reactions. The purities of the crosslinked ABD variants were controlled by RP-HPLC (Figure S2) analysis and were determined to be $\geq$95% for all variants except ABD_CL2 and ABD_CL3 (purity: 85 and 92%, respectively).

### Binding analysis

The HSA binding of the crosslinked versions of ABD and the ABD reference protein was analyzed in real time by SPR-based biosensor (ProteOn) experiments. The first screening experiment (Figure 3) showed that ABD_CL2 and ABD_CL12 had no affinity to HSA, whereas ABD_CL13 displayed weak binding.

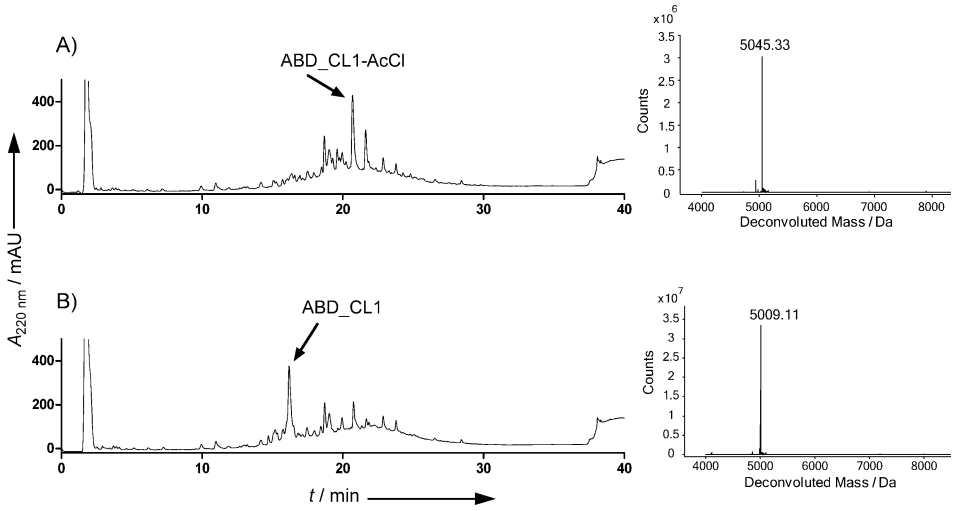


**Figure 2.** RP-HPLC analysis of the crude, synthetic protein ABD_CL1, with the ESI-MS analysis of the major peak shown to the right of each chromatogram. The chromatograms show the absorbance at 220 nm for A) linear, chloroacetylated ABD_CL1 before crosslinking, and B) the product formed after 3 h incubation in PBS pH 7.4, supplemented with 10 mM EDTA. Theoretical molecular weights: 5045.02 Da (ABD_CL1-AcCl) and 5008.52 Da (ABD_CL1).

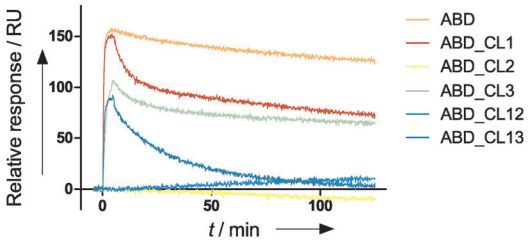


**Figure 3.** SPR sensorgrams showing the binding of ABD (reference) and crosslinked variants (50 nM) to HSA immobilized on a sensor chip.

ABD_CL3 showed a slower on-rate than the reference protein but a very slow off-rate, thus indicating high affinity. ABD_CL1 showed a fast on-rate and a two-phase dissociation curve: fast, immediate dissociation, followed by much slower dissociation after ~20 min.

The HSA binding of ABD_CL1 and the ABD reference protein was studied in more detail, but, due to the extremely slow off-rates of the ABD variants, it was not possible to use the ProteOn XPR36 for determination of the exact kinetic parameters. Thus, the calculated $K_D$ values (Table 1) can only be used for comparison in this series of experiments. The biosensor experiments were performed in triplicate and demonstrated that the crosslinked version ABD_CL1 binds to HSA with very high affinity ($K_D$ = 24 pM), which is comparable to the affinity of the reference protein ABD ($K_D$ = 21 pM).

**Table 1.** Kinetic parameters for binding to HSA.

| | $k_a$ [$M^{-1}s^{-1}$] | $k_d$ [$s^{-1}$] | $K_D$ [M] |
|---|---|---|---|
| ABD_CL1 | $(6.2\pm0.7)\times10^5$ | $(1.5\pm0.1)\times10^{-5}$ | $24\times10^{-12}$ |
| ABD | $(1.9\pm0.5)\times10^5$ | $(4.1\pm1.1)\times10^{-6}$ | $21\times10^{-12}$ |

## Circular dichroism spectroscopy

Circular dichroism was used to study the secondary structures and melting points of the ABD variants and reference protein. The CD spectra of ABD_CL1 and the ABD reference protein had local minima at 208 and 221 nm, characteristic of a predominantly α-helical secondary structure (Figure 4). The four other crosslinked variants did not show these characteristic spectra, thus indicating that their α-helical secondary structures were partly or completely lost (Figure S3).

Melting profiles were recorded for all variants by using CD signals at 221 nm. The melting profiles of ABD_CL1 and the reference protein showed a clear transition, thus indicating cooperative unfolding, characteristic of a well folded protein (Figure 5). The $T_m$ of ABD_CL1 (63 °C) was approximately 5 °C higher (reference protein $T_m$ 58 °C), thus indicating a more stable protein (Table 2).

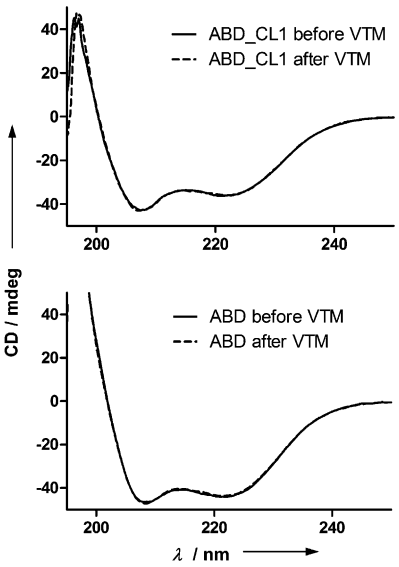


**Figure 4.** CD spectra of 50 μM ABD_CL1 (top) and ABD reference protein (bottom) at 20 °C before (——) and after (-----) variable temperature measurements.

## Proteolytic degradation

The first degradation screening experiments showed that ABD_CL1 had increased stability towards both pepsin and trypsin/chymotrypsin, compared to the reference protein (Fig-

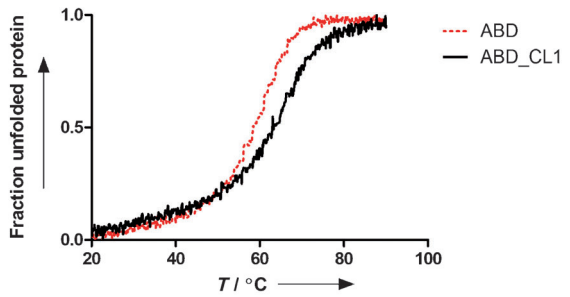


**Figure 5.** Thermal melting profiles of 50 μM ABD_CL1 and ABD reference protein.

**Table 2.** Summary of the properties of the ABD variants.

| Protein | Secondary structure | $T_m$ [°C] | HSA binding | Protease stability |
|---|---|---|---|---|
| ABD | +++ | 58 | ++++ | + |
| ABD_CL1 | +++ | 63 | +++ | ++++ |
| ABD_CL2 | – | n.a. | – | – |
| ABD_CL3 | + | 42 | ++ | – |
| ABD_CL12 | – | n.a. | – | – |
| ABD_CL13 | – | n.a. | + | – |

n.a.: No defined transition point was observed.

ures S4 and S5). None of the other variants showed improved protease stability under the same conditions.

The protease stabilities of ABD_CL1 and the reference protein were further studied over time. The protease stability of ABD_CL1 was dramatically improved compared to that of the reference protein (Figure 6), especially with pepsin. Under the conditions used, $t_{1/2}$ for pepsin degradation was 23 min for ABD_CL1 and 1 min for the reference protein; for trypsin/chymotrypsin $t_{1/2}$ was 10 min for ABD_CL1 and 2 min for the reference protein. When comparing AUCs (areas under the curve) it was seen that the total amount of intact, full-length protein during 60 min exposure was at least 17 times (1714%) higher for ABD_CL1 than for the reference protein in the presence of pepsin, and almost five times (482%) higher for trypsin/chymotrypsin. Both trypsin and chymotrypsin are found in pancreatin, and therefore usually act together in the degradation of proteins in the small intestine; however, individual protease experiments were performed with ABD_CL1 and the reference protein: ABD_CL1 was more stable when exposed to either trypsin or chymotrypsin alone (Figure S6).

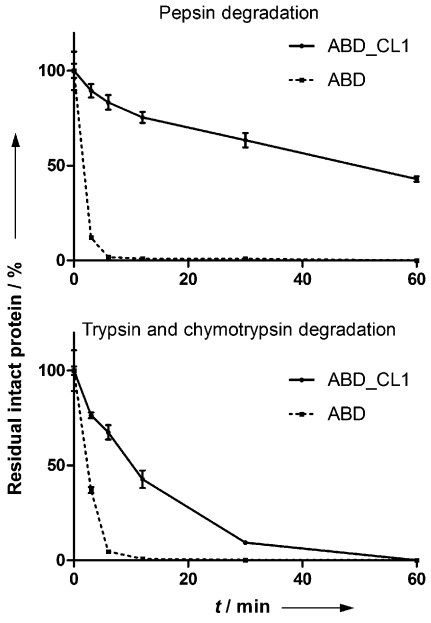


**Figure 6.** Proteolytic degradation of 5 μg ABD_CL1 and ABD reference protein by pepsin (0.5 μg mL$^{-1}$) at pH 2.6, 37 °C (top), and by trypsin (2.7 μg mL$^{-1}$) with chymotrypsin (1.2 μg mL$^{-1}$) at pH 7.4, 37 °C (bottom).

Investigation of the major pepsin degradation products of the reference protein by RP-HPLC and mass spectrometry showed that the peptide bond between Leu42 and Ala43 is rapidly cleaved, and that the C-terminal truncated protein was the dominating fragment after three minutes. This 42 aa fragment was then relatively quickly cleaved between Phe20 and Tyr21, thus giving rise to two new fragments (20 and 22 aa). When the reference protein was challenged with trypsin/chymotrypsin, the peptide bond between Tyr21 and Lys22 was the most susceptible (in addition, several fragments were detected for cleavage after arginine or lysine residues). These degradation patterns were not unexpected as pepsin preferentially cleaves at Phe, Tyr, Trp, and Leu in position P1 or P1′, whereas chymotrypsin preferentially cleaves at Trp, Tyr, and Phe in position P1, and trypsin preferentially cleaves at Arg or Lys at position P1.

## Discussion

Five thioether crosslinked ABD variants were synthesized. Circular dichroism analysis showed that only ABD_CL1 had a spectrum characteristic of an α-helical protein, but its helical content was slightly lower than for the ABD reference protein. The ABD_CL1 thioether bridge is between Cys17 (in the loop connecting helices 1 and 2) and Lys45 (in the C terminus). In the other variants a thioether bridge is between Cys1 or Lys5 (N terminus) and Cys28 or Lys29 (loop between helices 2 and 3; Figure 1). The NMR structure of the homologous, albumin-binding G148–GA3 domain shows that the N terminus is part of an α-helix; this suggests that this region is important for the correct folding of the three-helix structure and that side-chain modifications in this part of the protein are not compatible with folding.[33] In contrast, the C-terminal tripeptide (44–46) was found to be unstructured in the same study; this would explain the greater tolerance of side-chain modifications in this region.

Variable temperature measurements showed that ABD_CL1 $T_m$ was 5 °C higher than for the reference protein; the CD spectra of ABD_CL1 were identical before and after heating to 90 °C. These experiments demonstrated that the intramolecular thioether bridge was compatible with folding and that it increased thermal stability.

Interestingly, the secondary-structure content of the proteins did not fully correlate with affinity for binding to HSA, as deter-

mined by SPR biosensor analysis. ABD_CL3 (thioether bridge between Cys1 and Lys29) showed high affinity to HSA despite the low degree of helicity (CD analysis). The screening experiment demonstrated a slower on-rate for ABD_CL3 compared to the reference protein, thus suggesting that structural rearrangement is required for binding to HSA. Still, the slow off-rate shows that ABD_CL3 is capable of binding HSA with high affinity.

SPR-based binding kinetic analysis of ABD_CL1, which had the highest thermal stability, showed very high affinity for HSA. The dissociation curve showed a two-step behavior (one component with a fast off-rate, and one with a slow off-rate), thus suggesting that the analyte was heterogeneous and that there are two types of binding interaction with the immobilized ligand. It can be speculated that a fraction of the ABD_CL1 protein was not correctly folded and therefore bound with lower affinity to HSA; this would be in agreement with the lower helical content observed for ABD_CL1 compared to the reference protein. When the kinetic parameters were calculated for the association and dissociation phases, the equilibrium dissociation constants were found to be comparable for ABD_CL1 and the reference protein ($K_D \approx 20$ pM).

Proteolytic screening was performed with pepsin or a combination of trypsin and chymotrypsin. ABD_CL1 was more resistant to proteolytic degradation than the reference protein, whereas all other crosslinked variants were rapidly degraded under the same conditions. It is evident that correct folding of the protein is crucial for protease stability. This is not surprising, as a random polypeptide chain or a partially folded protein exhibiting high conformational flexibility would be expected to be more susceptible to the action of proteases than a compact, folded protein. It is clear that the introduction of an intramolecular thioether bridge in a correctly folded protein can give dramatically improved resistance to proteases. In this study, the amount of intact, crosslinked ABD_CL1 protein was 17 times higher during 60 min of pepsin treatment, compared to the reference protein.

The thioether crosslink between Cys17 and Lys45 in ABD_CL1 clearly makes the major cleavage sites in the protein less accessible to the investigated proteases. Pepsin cleavage between Leu42 and Ala43 (particularly efficient in the reference protein) was almost completely suppressed in ABD_CL1. Furthermore, the three investigated proteases showed efficient cleavage at Phe20-Tyr21-Lys22-Arg23 (in helix 2) of the reference protein, but this was suppressed in ABD_CL1. The protection of these major degradation sites in ABD_CL1 can be explained by the presence of the intramolecular thioether bridge close to the proteolytic sites; this is expected to sterically impede access of the proteases to the sensitive sites in the peptide backbone. However, it must be emphasized that the folded structure also seems to be crucial for increased proteolytic stability, as ABD_CL12 and ABD_CL13, which have both the Cys17–Lys45 thioether bridge and a second thioether bridge associated with loss of helical content, completely lost stability to the proteases.

Substitution of amino acid sequences susceptible to proteolytic cleavage is, in principle, a strategy to prevent the degradation. However, for the ABD protein, the region with the sensitive sequence (Phe20-Tyr21-Lys22-Arg23, including the major proteolytic sites for the investigated enzymes) is important for binding to HSA and could thus not be substituted without affecting the function of the protein. In an alanine scan of the interaction between a homologous ABD protein and HSA, the substitution of any of the five residues at positions 20–24 resulted in reduced affinity for HSA; the most dramatic decrease (86-fold increase of $K_D$) was found for the substitution of Tyr21 with Ala.[34] Thioether crosslinking could be a more generally applicable strategy for increasing proteolytic stability, as the protective effect seems to extend to sites outside the peptide bonds of the amino acids directly involved in the crosslinking reaction. It could also be expected to confer resistance to other proteases.

The chemoselective reaction used to produce the thioether bridges in this study relies on thioalkylation between a cysteine residue and a chloracetyl group coupled to the side chain of a lysine reside. With this strategy, the crosslinker length can easily be adjusted, for example, by using ornithine or diaminobutyric acid instead of lysine. The thioalkylation reaction is highly efficient (high yield with no detectable side reaction). The thioether bond is expected to be more stable than a disulfide bond to reducing conditions and modification by thiol, and could therefore be a better option for the stabilization of therapeutic proteins. For small proteins like the albumin-binding domain investigated in this study, introduction of the thioether bridge is straightforward; total chemical synthesis would be significantly more demanding for larger proteins. To explore the possibility of using thioether crosslinking with recombinantly produced proteins, an ABD variant was synthesized with two cysteine residues (positions 17 and 45) and reacted with a dibromide crosslinker (Figure S7). The protein crosslinked by this approach displayed high binding affinity to HSA (Figure S8) and CD spectra typical for $\alpha$-helical proteins, albeit with lower thermal stability than the ABD reference protein (Figure S9). When the crosslinked protein was tested with proteases, it was found to have higher resistance to pepsin than the reference protein, but the difference was not as dramatic as for ABD_CL1 (Figure S10). The proteolytic stability towards trypsin/chymotrypsin was only marginally better than for the reference protein. It can be concluded that although the crosslinked Cys17–Cys45 variant has an intramolecular bridge at the same position as for ABD_CL1, the crosslink is not sufficient to give the same degree of resistance to proteases; minor differences in the structure of the linker appear to be important. However, the observed increase in proteolytic stability still suggests that the concept could also be feasible for the stabilization of recombinantly produced proteins.

The ABD protein investigated in this study has clinical relevance as it could be used as fusion partner to increase the half-life of therapeutic peptides and proteins (by association with albumin). Although the most proteolytically stable variant requires chemical synthesis, this does not preclude its use as protein fusion partner, as the synthetic protein can easily be ligated to other peptides or proteins (recombinant or synthetic) by such methods as native chemical ligation (NCL, reviewed in

ref. [35]), expressed protein ligation (EPL, ref. [36]), or sortase A-mediated enzymatic protein ligation.[37,38] Importantly, the ABD protein can be considered a model of a small, therapeutic protein that in itself could have a pharmacological effect. As passage across the intestinal membrane and oral uptake have been demonstrated for certain classes of small, conformationally constrained proteins,[39] the application of chemical crosslinking to other small proteins of therapeutic relevance could be a general strategy for increasing the bioavailability of these protein drugs.

## Conclusion

This study shows that intramolecular crosslinking by site-specific introduction of a thioether bridge in an albumin-binding protein domain can be used to increase proteolytic stability towards the major intestinal proteases: pepsin, trypsin, and chymotrypsin. In addition to its superior proteolytic stability, the ABD_CL1 variant showed high thermal stability and retained high affinity for binding to HSA. Engineering by chemical modifications is proposed as a general strategy for the development of small, therapeutic proteins better suited for oral administration.

## Experimental Section

**Intramolecular crosslinking:** Crude synthetic peptide (ABD_CL1, ABD_CL2, ABD_CL3, ABD_CL12 or ABD_CL13) produced by using standard solid-phase peptide synthesis (see the Supporting Information) was dissolved (2 mg mL$^{-1}$) in standard PBS (10 mM $PO_4^{3-}$, 137 mM NaCl, and 2.7 mM KCl; pH 7.4) supplemented with ethylenediaminetetraacetic acid (10 mM, EDTA; Merck), and the reaction was allowed to proceed for 3 h or overnight (~18 h). For small-scale (~2 mg) crosslinking reactions, the reaction mixture was diluted (1:1) in TFA (0.2%)/$CH_3CN$ (40%) and purified by RP-HPLC. For large-scale reactions (10–20 mg), the buffer was exchanged to ammonium acetate (25 mM, pH 6) on PD-10 columns (GE Healthcare); the product was lyophilized, dissolved in TFA (0.1%)/$CH_3CN$ (20%), and purified by semipreparative RP-HPLC (Zorbax 300SB C18 column, 9.4×250 mm, 5 μm particle size; Agilent Technologies) on a 1200 series instrument (Agilent Technologies), and analyzed by LC-ESI-MS.

**Circular dichroism spectroscopy:** The proteins were dissolved (50 μM, ~0.25 mg mL$^{-1}$) in standard PBS (10 mM $PO_4^{3-}$, 137 mM NaCl, and 2.7 mM KCl; pH 7.4), and CD spectra and melting curves were recorded on a J-810 spectropolarimeter (path length 1 mm, 250–195 nm, 20 °C; Jasco), both before and after heating the sample to 90 °C. To determine $T_m$, circular dichroism (221 nm) was recorded while gradually increasing the temperature from 20 to 90 °C.

**SPR biosensor analysis:** The biosensor binding analyses were performed on a ProteOn XPR36 instrument (Bio-Rad, Hercules, CA) at 25 °C with PBST running buffer (NaCl (150 mM), $Na_2HPO_4$ (8 mM), $NaH_2PO_4$ (2 mM), Tween20 (0.005%), pH 7.4) at a flow rate of 50 μL min$^{-1}$. Injection of HCl (30 μL, 20 mM; 100 μL min$^{-1}$) was used for chip regeneration. In screening experiments for binding to HSA, ABD variants were diluted (50 nM) in running buffer and injected over the chip surface (2500 RU immobilized HSA). The association was studied for 300 s, and the dissociation was followed for 7200 s (2 h). For data analysis all samples were double referenced: the signal from a blank surface was first subtracted from the sample signal, then the signal from an injection of PBST (running buffer) over the HSA surface was subtracted.

The kinetics for the binding of ABD_CL1 and the ABD reference protein to HSA were studied by using a sensor chip with HSA immobilized on three different surfaces (1100–1600 RU). The proteins were diluted in running buffer (0.8, 1.6, 3.3, and 6.6 nM for ABD_CL1; 1.0, 2.0, 4.0, and 8.0 nM for reference ABD) and injected over the three surfaces, thus giving binding curves in triplicate. The association time was 300 s, and dissociation was followed for 36000 s (10 h).

The kinetic parameters for the binding were obtained by fitting the sensorgrams to a 1:1 Langmuir binding model in ProteOn Manager software (version 3.1.0.6; Bio-Rad) to obtain the average association rate constant ($k_a$ [M$^{-1}$s$^{-1}$]), dissociation rate constant ($k_d$ [s$^{-1}$]), and standard deviation; from this the dissociation equilibrium constant ($K_D$ [M]) was calculated ($K_D = k_d/k_a$).

**Digestion assay:** Proteolytic degradation of ABD and variants was studied for pepsin (3200–4500 U mg$^{-1}$, from porcine gastric mucosa; Sigma–Aldrich) and a mixture of trypsin (≈37 U mg$^{-1}$, from bovine pancreas; Sigma–Aldrich) and chymotrypsin (≥ 40 U mg$^{-1}$; from bovine pancreas, Sigma–Aldrich) with ABD protein (5 μg, 0.25 mg mL$^{-1}$ (50 μM) in PBS (pH 7.4); see details below).

**For challenging ABD protein with pepsin:** ABD protein (5 μg) was transferred to a microcentrifuge tube. Pepsin in HCl (10 mM, 32 μL) was added, thus resulting in ~pH 2.6 and a final pepsin concentration of 2 μg mL$^{-1}$ for screening and 0.5 μg mL$^{-1}$ for studying the degradation over time. In the screening experiment the samples were incubated for 10 min at 37 °C before enzyme inactivation by addition of NaOH (5 μL, 0.1 M, thus raising to >pH 9), and placing the sample on ice. A reference sample of each protein was inactivated with NaOH before pepsin was added to the tube. The degradation over time was monitored for ABD_CL1 and the ABD reference protein by incubation for 3, 6, 12, 30, or 60 min at 37 °C before inactivation with NaOH (or inactivated with NaOH before pepsin was added).

**For challenging ABD protein with trypsin and chymotrypsin:** ABD protein (5 μg) was transferred to a microcentrifuge tube. Trypsin and chymotrypsin in PBS pH 7.4 (32 μL) were added (final: 2.7 μg mL$^{-1}$ trypsin, 1.2 μg mL$^{-1}$ chymotrypsin). In the screening experiment the samples were incubated for 10 min at 37 °C before enzyme inactivation by addition of TFA (3 μL, 10%) and placing the sample on ice. A reference sample of each protein was inactivated with TFA before trypsin and chymotrypsin were added. Degradation over time was monitored for ABD_CL1 and the ABD reference protein by incubation for 3, 6, 12, 30, or 60 min at 37 °C before inactivation with TFA (or inactivated with TFA before trypsin and chymotrypsin were added).

Control experiments (ABD_CL1 or reference protein incubated with trypsin or chymotrypsin) were performed as described for the chymotrypsin/trypsin combination, but with only trypsin or chymotrypsin (final: 2.7 or 1.2 μg mL$^{-1}$, respectively), and for 0, 3, or 12 min.

All samples were analyzed by RP-HPLC on an analytical Zorbax 300SB C18 column (gradient: 15–45% B; A: aqueous TFA (0.1%); B: TFA (0.1%) in $CH_3CN$) over 25 min at 1.2 mL min$^{-1}$ by LC-ESI-MS on a 6520 Accurate Mass Q-TOF LC/MS instrument (Agilent Technologies).

Elution peaks for intact ABD protein were integrated, and AUC was calculated. The degradation experiments over time were performed in triplicate (trypsin and chymotrypsin controls in duplicate). The integrated area for each sample was normalized to the mean area of the $t=0$ samples for that variant; mean and standard error of the mean were calculated for each time point. For the control experiments, two replicates and the mean were plotted.

To quantify the stability improvement of the crosslinked variants, the AUC from the normalized charts were calculated for the three investigated variants and compared to the AUC of the reference ABD protein. The half-life ($t_{1/2}$) for ABD_CL1 and the ABD reference protein was calculated by fitting the data to a nonlinear one-phase decay in Prism 5 software (GraphPad, La Jolla, CA).

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[1] B. Leader, Q. J. Baca, D. E. Golan, *Nat. Rev. Drug Discovery* **2008**, *7*, 21 – 39.
[2] P. J. Carter, *Exp. Cell Res.* **2011**, *317*, 1261 – 1269.
[3] D. S. Dimitrov, *Methods Mol. Biol.* **2012**, *899*, 1 – 26.
[4] W. Jiskoot, T. W. Randolph, D. B. Volkin, C. R. Middaugh, C. Schöneich, G. Winter, W. Friess, D. J. A. Crommelin, J. F. Carpenter, *J. Pharm. Sci.* **2012**, *101*, 946 – 954.
[5] Y. Yun, Y. W. Cho, K. Park, *Adv. Drug Delivery Rev.* **2013**, *65*, 822 – 832.
[6] K. Park, I. C. Kwon, K. Park, *React. Funct. Polym.* **2011**, *71*, 280 – 287.
[7] R. M. Reilly, R. Domingo, J. Sandhu, *Clin. Pharmacokinet.* **1997**, *32*, 313 – 323.
[8] J. Swaminathan, C. Ehrhardt, *Expert Opin. Drug Delivery* **2012**, *9*, 1489 – 1503.
[9] J. Renukuntla, A. D. Vadlapudi, A. Patel, S. H. S. Boddu, A. K. Mitra, *Int. J. Pharm.* **2013**, *447*, 75 – 93.
[10] L. Illum, *J. Controlled Release* **2012**, *161*, 254 – 263.
[11] F. Depreter, G. Pilcer, K. Amighi, *Int. J. Pharm.* **2013**, *447*, 251 – 280.
[12] J. A. Getz, J. J. Rice, P. S. Daugherty, *ACS Chem. Biol.* **2011**, *6*, 837 – 844.
[13] D. J. Craik, R. J. Clark, N. L. Daly, *Expert Opin. Invest. Drugs* **2007**, *16*, 595 – 604.
[14] F. Zoller, T. Schwaebel, A. Markert, U. Haberkorn, W. Mier, *ChemMedChem* **2012**, *7*, 237 – 247.
[15] D. J. Craik, A. C. Conibear, *J. Org. Chem.* **2011**, *76*, 4805 – 4817.
[16] M. Werle, K. Kafedjiiski, H. Kolmar, A. Bernkop-Schnürch, *Int. J. Pharm.* **2007**, *332*, 72 – 79.
[17] M. Werle, T. Schmitz, H.-L. Huang, A. Wentzel, H. Kolmar, A. Bernkop-Schnürch, *J. Drug Targeting* **2006**, *14*, 137 – 146.
[18] J. Herrmann, R. Bodmeier, *Drug Dev. Ind. Pharm.* **2003**, *29*, 1027 – 1033.
[19] M. Muttenthaler, A. Andersson, A. D. de Araujo, Z. Dekan, R. J. Lewis, P. F. Alewood, *J. Med. Chem.* **2010**, *53*, 8585 – 8596.
[20] R. Rink, A. Arkema-Meter, I. Baudoin, E. Post, A. Kuipers, S. A. Nelemans, M. H. J. Akanbi, G. N. Moll, *J. Pharmacol. Toxicol. Methods* **2010**, *61*, 210 – 218.
[21] L. D. Kluskens, S. A. Nelemans, R. Rink, L. de Vries, A. Meter-Arkema, Y. Wang, T. Walther, A. Kuipers, G. N. Moll, M. Haas, *J. Pharmacol. Exp. Ther.* **2009**, *328*, 849 – 854.
[22] P. J. Knerr, A. Tzekou, D. Ricklin, H. Qu, H. Chen, W. A. van der Donk, J. D. Lambris, *ACS Chem. Biol.* **2011**, *6*, 753 – 760.
[23] G. N. Moll, A. Kuipers, L. de Vries, T. Bosma, R. Rink, *Drug Discovery Today Technol.* **2009**, *6*, e13 – e18.
[24] H. Jo, N. Meinhardt, Y. Wu, S. Kulkarni, X. Hu, K. E. Low, P. L. Davies, W. F. DeGrado, D. C. Greenbaum, *J. Am. Chem. Soc.* **2012**, *134*, 17704 – 17713.
[25] A. Angelini, P. Diderich, J. Morales-Sanfrutos, S. Thurnheer, D. Hacker, L. Menin, C. Heinis, *Bioconjugate Chem.* **2012**, *23*, 1856 – 1863.
[26] T. Ekblad, V. Tolmachev, A. Orlova, C. Lendel, L. Abrahmsén, A. E. Karlström, *Biopolymers* **2009**, *92*, 116 – 123.
[27] F. Y. Frejd in *Therapeutic Proteins: Strategies to Modulate Their Plasma Half-Lives* (Ed.: R. Kontermann), Wiley-VCH, Weinheim, **2012**, pp. 269 – 283.
[28] A. Jonsson, J. Dogan, N. Herne, L. Abrahmsén, P.-A. Nygren, *Protein Eng. Des. Sel.* **2008**, *21*, 515 – 527.
[29] V. Tolmachev, A. Orlova, R. Pehrson, J. Galli, B. Baastrup, K. Andersson, M. Sandström, D. Rosik, J. Carlsson, H. Lundqvist, A. Wennborg, F. Y. Nilsson, *Cancer Res.* **2007**, *67*, 2773 – 2782.
[30] A. Orlova, A. Jonsson, D. Rosik, H. Lundqvist, M. Lindborg, L. Abrahmsen, C. Ekblad, F. Y. Frejd, V. Tolmachev, *J. Nucl. Med.* **2013**, *54*, 961 – 968.
[31] J. T. Andersen, R. Pehrson, V. Tolmachev, M. B. Daba, L. Abrahmsén, C. Ekblad, *J. Biol. Chem.* **2011**, *286*, 5234 – 5241.
[32] O. E. Levy, C. M. Jodka, S. S. Ren, L. Mamedova, A. Sharma, M. Samant, L. J. D'Souza, C. J. Soares, D. R. Yuskin, L. J. Jin, D. G. Parkes, K. Tatarkiewicz, S. S. Ghosh, *PLoS One* **2014**, *9*, e87704.
[33] M. U. Johansson, I.-M. Frick, H. Nilsson, P. J. Kraulis, S. Hober, P. Jonasson, M. Linhult, P.-A. Nygren, M. Uhlén, L. Björck, T. Drakenberg, S. Forsén, M. Wikström, *J. Biol. Chem.* **2002**, *277*, 8114 – 8120.
[34] M. Linhult, H. K. Binz, M. Uhlén, S. Hober, *Protein Sci.* **2002**, *11*, 206 – 213.
[35] P. E. Dawson, S. B. H. Kent, *Annu. Rev. Biochem.* **2000**, *69*, 923 – 960.
[36] T. W. Muir, *Annu. Rev. Biochem.* **2003**, *72*, 249 – 289.
[37] H. Mao, S. A. Hart, A. Schink, B. A. Pollok, *J. Am. Chem. Soc.* **2004**, *126*, 2670 – 2671.
[38] T. Proft, *Biotechnol. Lett.* **2010**, *32*, 1 – 10.
[39] C. T. T. Wong, D. K. Rowlands, C.-H. Wong, T. W. W. Lo, G. K. T. Nguyen, H.-Y. Li, J. P. Tam, *Angew. Chem. Int. Ed.* **2012**, *51*, 5620 – 5624; *Angew. Chem.* **2012**, *124*, 5718 – 5722.

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